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Our overall objective is to understand how dysregulation of cell migration contributes to tumor cell invasiveness in prostate cancer. A combination of correlative epidemiological studies and basic experimental investigations demonstrate a role for upregulated EGF receptor (EGFR) signaling of motility in tumor progression. EGFR-mediated cell motility has been demonstrated to be critical for tumor invasion. *Our central premise is that prostate tumor cell invasiveness can be inhibited by interfering with the specific motility-associated calpain activation that governs the critical underlying biophysical process of de-adhesion.* Prior work by ourselves and others has shown that integrin/matrix binding and growth factor stimulation jointly regulate cell locomotion. These studies have identified cell/substratum adhesiveness, especially the ability of a cell to detach at its trailing edge, as a primary governor of cell locomotion. We have recently found that this tail detachment is regulated by calpain activation. We are employing a set of model prostate tumor cell lines including the moderately invasive androgen-independent PC3 cell and its highly metastatic variant PC3M cell, along with a panel of syngeneic androgen-independent DU-145 cells that vary in invasiveness. Our findings in the first year show that disruption of calpain activation and de-adhesion can block tumor invasiveness.

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CELL MOTILITY IN TUMOR INVASION

Alan Wells, Douglas Lauffenburger, Timothy Turner

INTRODUCTION

Our overall objective is to understand how dysregulation of cell migration contributes to tumor cell invasiveness in prostate cancer. A combination of correlative epidemiological studies and basic experimental investigations demonstrate a role for upregulated EGF receptor (EGFR) and other receptor signaling of motility in tumor progression. Especially in prostate tumor cells, EGFR-mediated cell motility has been demonstrated to be critical for tumor invasion. Since signals from extracellular matrix through integrins and from cell-cell contacts also strongly influence cell motility, the underlying common biophysical processes and biochemical controls of motility offer an attractive target for limiting tumor progression.

Our central premise is that prostate tumor cell invasiveness can be inhibited by interfering with the specific motility-associated calpain activation that governs the critical underlying biophysical process of de-adhesion. Prior work by ourselves and others has shown that integrin/matrix binding and growth factor stimulation jointly regulate cell locomotion. These studies have identified cell/substratum adhesiveness, especially the ability of a cell to detach at its trailing edge, as a primary governor of cell locomotion. We have recently found that this tail detachment is regulated by calpain activation. We will employ a set of model prostate tumor cell lines including the moderately invasive androgen-independent PC3 cell and its highly metastatic variant PC3M cell, along with a panel of syngeneic androgen-independent DU-145 cells that vary in invasiveness. We will determine whether targeted disruption of calpain activation and de-adhesion can block tumor invasiveness.

BODY

The original Statement of Work (Table 1) described a series of tasks to accomplish the two Objectives proposed and the additional training Objective. We have tackled these Tasks in the order of greatest yield so that work in areas can progress as systems are being optimized in others. The main efforts during the first year of this three-year project have been focused on the prostate tumor cell motility and invasion efforts and developing trainees. The progress during this first year has put us in good position to accomplish the tasks within the time-frame provided.

Table 1. Original Statement of Work

Work to be performed at University of Pittsburgh (A. Wells Laboratory):

1. determine whether calpain is activated by growth factors and integrins in prostate cancer cells
2. determine whether calpain is limiting for prostate tumor cell motility on complex surfaces
3. determine whether prostate tumor cell transmigration of extracellular matrices is dependent on calpain activity
4. determine whether inhibition of calpain limits tumor invasiveness and metastasis in murine models of progressive prostate cancer

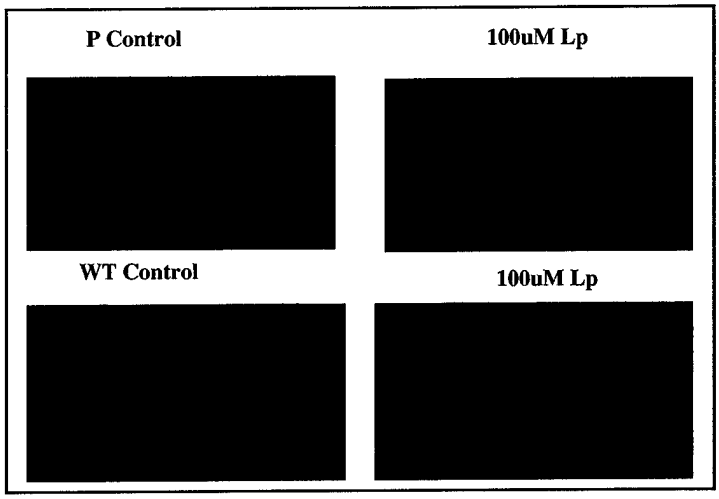
Work to be performed at MIT (D.A. Lauffenburger Laboratory):

1. determine optimal adhesiveness and high and low adhesiveness surfaces for fibroblast motility
2. test prostate tumor cell motility on defined adhesiveness surfaces
3. determine whether calpain activation is required for prostate cell motility

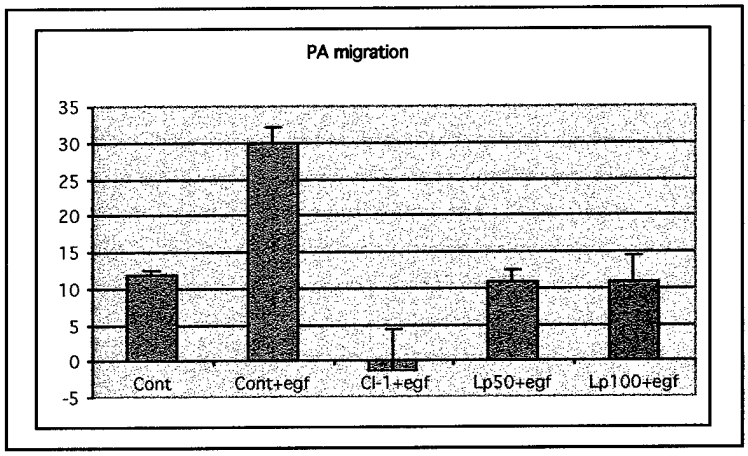
Work to be performed in partnership with Tuskegee (T. Turner Laboratory):

1. trainees will perform prostate cell growth and motility assays at Tuskegee and UPitt
2. trainees will perform in vivo mouse assays at UPitt

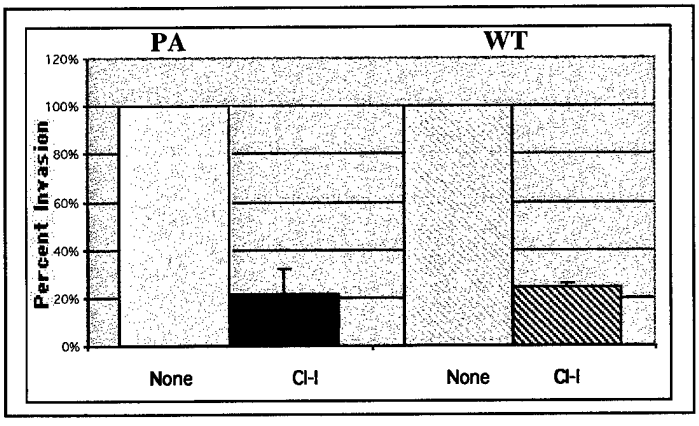
Work to be performed at University of Pittsburgh:



Task 1. determine whether calpain is activated by growth factors and integrins in prostate cancer cells. We have begun to tackle this task. Initial findings using the live cell BOC assay (calpain-mediated cleavage scores as blue) to assess activation of calpain in live cells demonstrate that EGF induces calpain in the DU-145 cells, both WT and Parental (Fig 1). The specificity of activation is shown by inhibition by calpain inhibitor I (not shown) and leupeptin. We do not yet have data on integrin stimulation of calpain in these cells; this should be examined in years 2/3.



Task 2. determine whether calpain is limiting for prostate tumor cell motility on complex surfaces. Our initial data demonstrate that calpain inhibitor I and leupeptin can limit DU-145 motility across self-generate matrix (Fig 2). This suggests that calpain can be targeted to limit tumor cell invasion by blocking migration. During years 2/3 we expect to examine motility on define complex matrices.

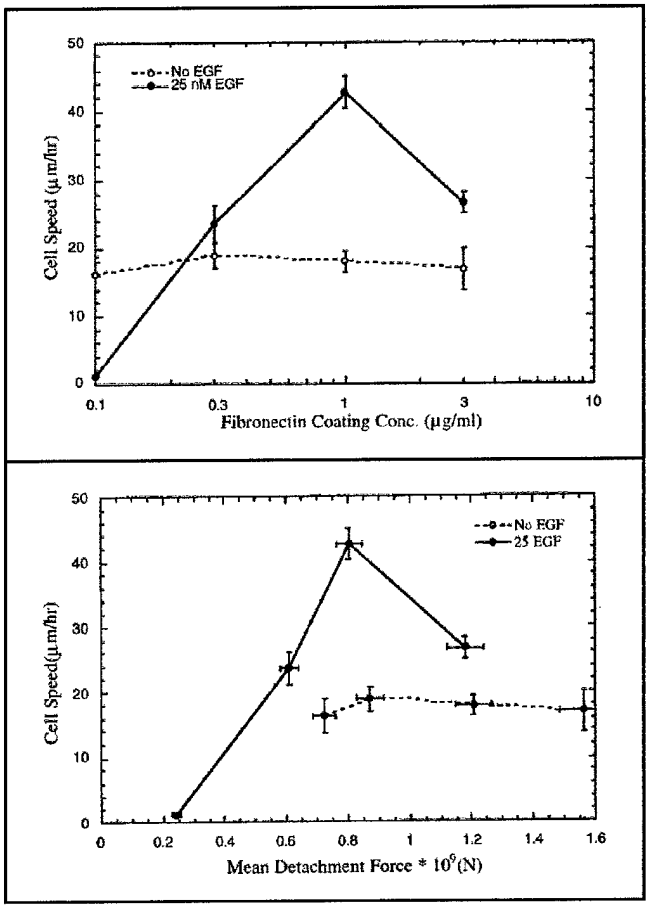


Task 3. determine whether prostate tumor cell transmigration of extracellular matrices is dependent on calpain activity. The first part of this task has been completed. In vitro transmigration of a Matrigel matrix by both Parental and WT EGFR-expressing DU-145 cells is blocked by inhibitors of calpain, CI-I and leupeptin (not shown) (Fig 3). Furthermore, antisense downregulation of M-calpain limits this transmigration, providing specificity (not shown).

Task 4. determine whether inhibition of calpain limits tumor invasiveness and metastasis in murine models of progressive prostate cancer. We have challenged mice with DU-145 prostate carcinoma tumor xenografts with inhibitors of calpain. Tumor invasiveness was reduced in the presence of daily injections of the inhibitor leupeptin (Table 1). The differences invasiveness between treated and mock treated were significant ($P < 0.05$) by T-test and ANOVA analyses.

	WT+HBSS	WT+Leupetin	Parental+HBSS	Parental+leupeptin
Diaphragm invasiveness	2.06	1.33	1.78	0.72
Diaphragm tumors	14 /14	13 /14	14 /15	11 /14

Work to be performed at MIT:
Task 5. determine optimal adhesiveness and high and low adhesiveness surfaces for fibroblast motility. This is an ongoing project that derives from our previous work on integration of integrin and growth factor signaling of motility. For EGF-induced motility, we find that optimal fibronectin coating occurs at around 1ug/ml, with 0.3 ug/ml and 3 ug/ml being low and high adhesiveness, respectively. At these extremes, motility is reduced to levels on par with no EGF stimulation (Fig 4). The optimal adhesive strength of the fibroblasts to the surface is approximately 0.8 nN, with a movement of 0.2 nN either direction wiping out growth factor induced movement (Fig 5).



Task 6. test prostate tumor cell motility on defined adhesiveness surfaces. During years 2/3 DU-145 cell motility will be tested on these fibronectin coated surfaces to determine the biphasic response of adhesion to motility. This will be tested in the presence and absence of EGFR inhibitors to account for autocrine signaling.

Task 7. determine whether calpain activation is required for prostate cell motility. On the basis of the above and findings in Task 2, we will pursue this vigorously in years 2/3.

Work to be performed in partnership with Tuskegee:
Task 8. trainees will perform prostate cell growth and motility assays at Tuskegee and UPitt. Masters students Clayton Yates and Karlyn Bailey have been trained at Tuskegee to perform these assays with the DU-145 human prostate tumor lines. Clayton Yates has transitioned to

University of Pittsburgh as a PhD degree student in the Cellular and Molecular Pathology graduate program. Most of his first year were spent on completing required coursework and passing the Comprehensive Exam necessary to be accepted to a degree-granting program and selecting a thesis laboratory (Wells laboratory).

Task 9. trainees will perform in vivo mouse assays at UPitt. During his rotation in the Wells laboratory (all PhD students at University of Pittsburgh School of Medicine are required to complete three laboratory rotations prior to selecting a graduate program and thesis laboratory), Mr. Yates learned the in vivo mouse tumor growth and invasion assays. He will apply these in years 2/3.

KEY RESEARCH ACCOMPLISHMENTS

- EGFR signaling enhances prostate tumor motility
- EGFR signaling increases calpain activity in prostate cancer cells
- Calpain inhibitors block prostate tumor invasiveness in vitro
- Calpain inhibitors block prostate tumor invasiveness in vivo
- EGFR signaling enhances fibroblast motility over a narrow range of fibronectin adhesiveness
- One trainnee successfully transitioned from Tuskegee Masters program to a doctoral program at University of Pittsburgh

REPORTABLE OUTCOMES

Articles:

A Glading, DA Lauffenburger, A Wells (2002). Cutting to the chase: calpain proteases in cell motility. Trends in Cell Biology 12, 46-54. (appended)

A Wells, J Kassis, J Solava, T Turner, DA Lauffenburger (2002). Growth factor-induced cell motility in tumor invasion. Acta Oncologica 41, 124-130. (appended)

Abstracts:

A Mamoune, J Kassis, D Lauffenburger, A Wells (2002) Calpain inhibition reduces prostate tumor invasion. American Association for Cancer Research (AACR) Annual Meeting, San Francisco, CA

Clayton C. Yates, Karlyn J. Bailey, Alan Wells and Timothy Turner (2001). The Effects of the Luteinizing Hormone Releasing Hormone Antagonist, Cetrorelix on the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Selected Abstract-5th Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association, Maui, HI

Clayton C. Yates, Karlyn J. Bailey, Alan Wells and Timothy Turner (2001). Cetrorelix, a Luteinizing Hormone Releasing Hormone Antagonist, Influences the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Selected Abstract-Keystone Symposium, Tahoe City, CA

Manuscript in preparation:

A Mamoune, J Kassis, D Lauffenburger, A Wells (2002) Calpain inhibition decreases tumor invasion of human prostate cancer cells. In preparation for submission

Training:

C Yates has transitioned from Tuskegee University with a MA in Biology to the PhD program in Cellular and Molecular Pathology at University of Pittsburgh.

CONCLUSIONS

The initial year of this multiyear award has reached major defined milestones and established the base for increasing productivity over the life of the award.

Importance/Implications: The Key Accomplishments above firmly demonstrate the validity of the model of the tumor biology that calpain-mediated deadhesion is a rate-limiting step in tumor cell motility and invasion. This provide the 'proof a concept' that targeting calpain is a rationale therapeutic option. The implications are clear that calpain inhibitors, currently being developed for muscle-wasting conditions, may have a role as adjuvant cancer therapy to limit the spread of prostate carcinoma.

Recommended changes: The results to-date do not cause us to re-evaluate the Task list or overall thrust of the work. We will proceed with the unfinished Tasks in years 2/3, barring new data that might accrue.

University of Pittsburgh

Institutional Animal Care & Use Committee

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412-383-2008
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University of Pittsburgh Protocol Number: 0205438

May 16, 2002

NIH - NCI

Assurance Number: A3187-01

To Whom It May Concern:

The Institutional Animal Care and Use Committee of the University of Pittsburgh has reviewed and approved on May 16, 2002 the research proposal submitted by Alan Wells, PhD.

Titled: CELL MOTILITY IN TUMOR INVASION - Supplement.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

H Edwin Kennah

H. Edwin Kennah, Ph.D., Director
Institutional Animal Care and Use Committee

REVIEW ARTICLE

Taylor&Francis
healthsciences

Growth Factor-Induced Cell Motility in Tumor Invasion

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Tumor progression to the invasive and metastatic states dramatically enhances the morbidity and mortality of cancer. Rational therapeutic interventions will only be possible when we understand the molecular mechanisms governing the cell behavior underlying this transformation. For invasion, a subpopulation of tumor cells must recognize the extracellular matrix barrier, modify the barrier, migrate through the barrier, and then proliferate in the adjacent but ectopic locale. Prevention of any one of these steps would prevent invasion, but determining the most sensitively dysregulated step should provide the most promising therapeutic index. In many invasive tumors, upregulation of active motility is stimulated by growth factor receptor signaling, the EGF receptor being the most frequently implicated. Two key downstream molecular switches, PLCγ and m-calpain, are required for growth factor-induced motility but not basal, matrix-stimulated motility. Inhibition of either of these enzymes blocks in vitro and in vivo invasion of prostate, breast, and bladder carcinomas and glioblastomas. These represent novel and potentially selective targets for drug development. Future advances in the imaging of tumors in animals and ex vivo organ culture systems should provide additional new targets.

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Tumor invasion into and destruction of adnexa account for significant morbidity and mortality in a variety of tumors, particularly glioblastomas and carcinomas of the prostate, bladder, head and neck, and esophagus. These primary tumors are accessible for debulking by surgical and radiological means but local extension beyond the physiological borders render these approaches impotent or engender significant adverse effects and poor outcomes in themselves. Limiting further invasion may yield palliative benefit, delay further tissue destruction, or even stabilize the disease at an advanced but manageable stage. Thus, the control of invasion per se is an appropriate goal for cancer treatment, to be employed in conjunction with other approaches that target tumor cell proliferation. However, to target this aspect of tumor progression rationally, greater understanding of the operative molecular controls is required.

Growth factor receptors originally were linked to tumorigenesis, as a number of retroviral oncogenes were found to derive from peptide growth factors and their receptors; the primary examples were the v-erbB/EGF receptor and v-sis/PDGF. In the immediate post-proto-oncogene era, a wide variety of human tumors were found to overexpress this class of signaling molecules, the epidermal growth factor (EGF) receptor (EGFR) being the most frequently identified (1). However, these early studies in all likelihood underestimated this relationship since they targeted gene amplification and steady-state protein levels. The vast majority of epithelial cells express EGFR while producing ligands for this receptor, primarily EGF and TGFα, though these are spatially segregated by cell polarization of receptor to basolateral surfaces and ligand released at the apical membranes to prevent autocrine signaling (2, 3). When the epithelium becomes dysplastic and neoplastic with weakened cell–cell junctions, the segregation is lost and inappropriate autocrine signaling is enabled (4). As receptors and ligands are actively internalized and degraded upon binding and receptor activation, misleadingly low receptor protein levels may be found at steady state for highly active signaling loops (5). This situation was the reason for earlier controversies in prostate cancers in which various reports failed reproducibly to detect EGFR; however, examination of mRNA

Paper presented at the 'State of the Art' Conference on Cancer, Swedish Cancer Society, Stockholm, August 2001.

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species demonstrated high levels of both receptor and ligands (6). Thus autocrine signaling through the EGF receptor system is likely a common occurrence in carcinomas.

The prevalence of upregulated EGFR signaling in tumors did not initially provide insight into how this might contribute to tumor development. Upon closer examination of the clinical data, a number of studies demonstrated that EGFR overexpression correlated not with proliferation and initial tumorigenesis but, rather, with tumor progression to invasion. Sentinel examples include glioblastomas, in which half or more of the invasive tumors present upregulated EGFR compared to almost none of the non-invasive gliomas (7, 8). In one study of bladder carcinomas, most (21/24) of the invasive but few of the superficial (7/24) tumors overexpressed EGFR (9). High levels of EGFR were visualized in the regions of the tumors that were actively invading the underlying musculature while the superficial aspects of the tumors expressed lower, physiological levels of EGFR (10). In a series of gastric carcinomas, approximately one-third of the invasive tumors (38/130) overexpressed EGFR, whereas none of the early superficial tumors (0/26) similarly upregulated this system (11). Thus, EGFR signaling was hypothesized to contribute to the invasive phenotype.

To simplify matters thoroughly, in order to invade, a subpopulation of tumor cells must acquire the abilities to (i) recognize the extracellular matrix (ECM) barrier, (ii) modify the ECM barrier, including proteolytic degradation, (iii) actively migrate through the matrix space, and (iv) proliferate in the ectopic but adjacent site (12). It is not required that all of these are upregulated during tumor progression, since a basal level of the first three occurs in localized tumors and even in normal tissue. Thus, while any of these properties could be, and have been targeted to block invasion, determination of one that might be most problematically dysregulated to drive invasion could provide insight regarding which might provide the greatest therapeutic index as a target.

CELL-MATRIX INTERACTIONS

Paradoxically, the initial step of altered adhesion is both the most elucidated and least dissected to date. One reason for this is that the action of recognizing the barrier matrix is not passive but induces changes in cell behavior, often inducing the other properties needed for invasion. A second reason is that the effects of matrix may be non-monotonic or -monophasic for even a singular property, so that prediction of even the qualitative effect of altering a recognition effect is difficult. A third reason is that the cell-matrix interaction is highly dynamic. In poorly differentiated and invasive carcinomas the matrix changes, including upregulation of fetal and wound response proteins such as tenascin-C and laminin-5 (13, 14). The re-emergence of these proteins provides new sites for interactions and with different integrins and other adhesion receptors. For all these reasons, cell-matrix interactions in motility and invasion are difficult to understand even qualitatively.

Even the same cellular receptors for the matrix qualitatively alter their behavior with tumor progression. One well-documented example is the $\alpha 6\beta 4$ integrin, which normally functions as an anchor to the substratum in well-differentiated epithelia (15). In invasive carcinoma cells, $\alpha 6\beta 4$ relocalizes and concentrates at the leading edge of the cells. These receptors now drive motility forward, even signaling from laminin-5, which is soluble, rather than matrix embedded (16). Thus, this integrin changes from primarily an anchoring function to one of active signaling. Furthermore, even the same integrin-ligand pair function in qualitatively and quantitatively distinct ways depending on the geometry of ligation. It is well established that cell shape as dictated by the adhesion footprint impacts on cell function (17) and that the extent of integrin clustering dictates which signals are induced (17). We have shown that cells can adhere to individual integrin ligands, but only microclusters will support both integrin-mediated haptokinesis and growth factor-induced chemokinesis (18).

The next step in the invasion program is to alter the matrix to allow passage. The major aspect of this process involves extracellular proteases (19). Inhibitors of metalloproteinases (MMP) block tumor progression but the underlying mechanism for this is undergoing reappraisal (20). It is unlikely to be simply matrix degradation, as non-invasive tumor cells and even normal cells express high levels of many MMPs; moreover, cell migration is not necessarily enhanced by matrix breakdown since adhesive traction can be lost. Rather, the membrane-tethered MMP (intrinsic transmembrane MT-MMP and secreted MMP bound to cell surface receptors) might function itself as a motility-promoting adhesion receptor at the tip of an invadipodia (21, 22), or the MMP might function to create signals for cell motility either by cleaving autocrine growth factors (23), releasing sequestered growth factors from the matrix, or uncovering cryptic signals in matrix components (24, 25) (Fig. 1).

This last possibility is an especially exciting new avenue. It had earlier been reported that matrix components might activate receptors that are members of the family of growth factor receptors with intrinsic tyrosine kinase activity (RPTK). The most striking example of this is the discoidin domain receptors that bind fibrillar collagen (26, 27). Another matrix component, decorin, has been shown to activate EGFR (28). This concept potentially has been extended recently with the report that some of the EGF-like repeats in the onco-fetal and wound repair protein tenascin-C can signal through the EGF receptor (25). These monomers are of very low affinity with a Kd in excess of 10 μM. However, this protein is produced as a hexamer with 84 repeats clustered centrally, enabling multivalent binding (14); When expressed multivalent on

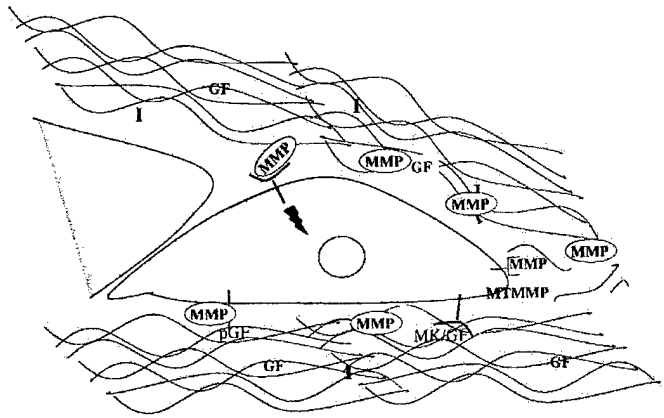

Fig. 1. Metalloproteinases (MMP) and other secreted proteases may promote motility by a number of avenues. The MMP were first hypothesized to digest the matrix (lines) at the front of the tumor edge to allow passage; this may be due to secreted and soluble MMP, secreted but receptor-bound MMP, or the membrane tethered MMP (MTMMP). Alternatively, MMP may signal through cell surface receptors (lightning bolt), liberate membrane tethered pro-growth factors (pGF), digest invasion inhibitors (I) in the matrix, liberate matrix-sequestered growth factors (GF), or uncover matrikines (MK/GF) that signal through members of the growth factor receptor superfamily.

beads the individual EGF-like repeats can form stable complexes with EGFR. This type of matricrine signaling through growth factor receptors greatly expands the regulatory functions of matrix components. However, this complexity of simultaneous adhesion, degradation and signaling confound clear analyses of the contribution of this stage to tumor invasion.

CELL MOTILITY

Cell motility should be central to tumor invasion since to grow in the ectopic site the cells must transmigrate a physiological barrier, ECM and, in the case of prostate and bladder carcinoma, enveloping muscular layer. However, in order directly to address this hypothesis, regulatory molecules need to be identified that are specific to the process of cell motility. Thus, inhibition of these signaling pathways should not affect other required cell properties such as proliferation.

Motility can be considered as a cyclic series of the biophysical processes of extension, front adhesion, transcellular contraction and rear release (29). All of this is preceded by reorganization of the actin cytoskeleton that enables normally cuboidal epithelial cells to assume the asymmetric fusiform shape that enables active locomotion (30). These steps are likely controlled by external signals acting through separable signaling pathways. Over the past decade, a large cohort of investigators has begun to identify key switches for motility in response to adhesion and growth factor signals (31) (Fig. 2). Growth factor-induced motility differs from basal adhesion-mediated motility not

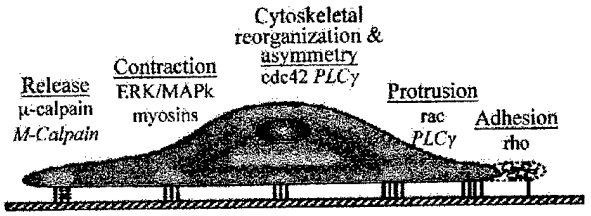

Fig. 2. Biophysical dissection of cell migration highlights key molecular switches. Cell movement can be considered as a series of distinct but concerted events, each of which involves separate signaling. As illustrated, each of these processes involves a unique set of effector molecules and signaling pathways. Required molecular switches for adhesion receptor-mediated motility are shown in block and those for growth factor receptor-mediated motility in italics, though the signaling separation may overlap somewhat. Taken with permission from Kassis et al. (57).

only quantitatively in generating a greatly enhanced rate but also in the fact that it is superimposed upon haptokinesis; while one might speculate that adhesion-signaled motility processes might be required (18), this has not yet been proven. Such a situation suggests that signaling pathways to growth factor-induced motility may have distinct switches, providing for a therapeutic window.

This has been borne out by our extirpation of two signaling pathways that appear highly selective for growth factor-induced motility. Briefly, activation of PLCγ is required for motility signaled by EGFR (32), PDGFR (33) and IGF-1R (34) but not integrins (35). PLCγ actuates motility by hydrolyzing PIP2 to mobilize gelsolin to sever and cap the actin cytoskeleton in the immediate submembrane region (36). This allows for the cytoskeletal reorganization necessary for cell polarization and continuous cytoskeletal plasticity required for the forward flow of the cell (37). Of importance for parsing events, PLCγ inhibition actually leads to increased proliferation not inhibition of growth by shunting the EGFR signaling towards that competitive cell response (38). Thus, PLCγ can be targeted as a motility-selective signal; in such a case other agents and/or approaches would need to be utilized to limit tumor cell growth.

A second key regulatory switch selective for growth factor-induced motility is the m-calpain isoform of this ubiquitous intracellular limited protease (39). Activation of this molecule is required for the lessened adhesion needed during tail retraction (40). The other ubiquitous isoform, μ-calpain, appears to be required during haptokinesis (41) and chemokine-driven motility (Satish et al. unpublished study, 2001). Currently, molecular inhibitors can differentially target these isoforms. Cell motility can be inhibited by pharmacological agents, such as ALLN, at levels that do not abrogate growth factor-induced proliferation. Thus, two switches that are selective for growth factor-induced motility provide targets to limit just this type of cell movement. This would minimize any deleterious side effects, since the integrin-mediated slow motility appears to be fully sufficient for homeostasis.

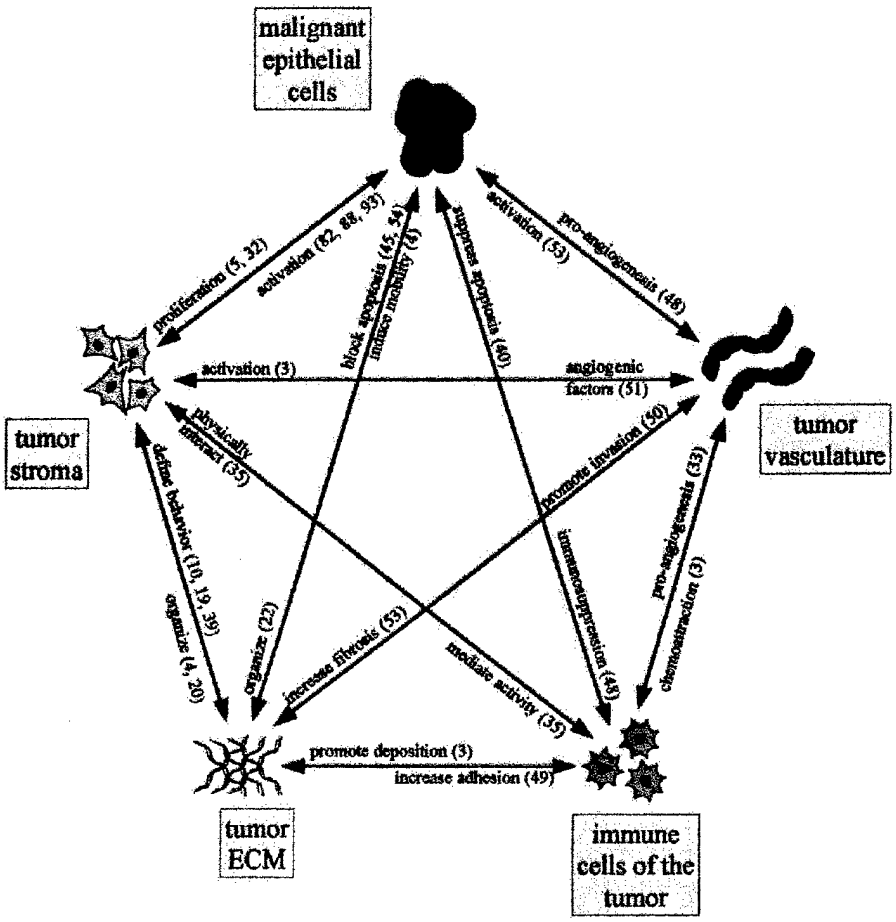

Fig. 3. Tumor progression is a multicellular field event involving matrix, and stromal and other support cells. The emerging concept of tumor multicellularity posits bi-directional effects of the neoplastic cells with the stromal cells, extracellular matrix, vasculature, and even immune response cells. Taken with permission from Radisky et al. (48).

With these tools, we could ask, at least for therapeutic targeting purposes, whether tumor invasion could be considered effectively as a disease of dysregulated EGFR-induced motility. In human prostate carcinoma, cell lines, overexpression of a motility-inducing EGFR construct but not a fully mitogenic but non-motile construct resulted in increased cell invasiveness in vitro and in vivo (42, 43). This invasiveness was due to autocrine EGFR signaling as inhibiting EGFR signaling with a selective pharmacological agent blocked the invasiveness of prostate, breast and bladder carcinomas (44). Pharmacological and molecular inhibitors of PLCγ prevented invasion of these cells into the diaphragm or other abdominal organisms when these tumor cells were grown intraperitoneally in athymic mice (43, 45). Such inhibitors also blocked invasion of glioblastoma cells into normal brain tissue in ex vivo model systems (46). This latter tumor type highlights another advantage of targeting the convergent molecular switches rather than the specific triggering receptor since it is an open question whether glioblastomas are driven to invade by EGFR, PDGFR, IGF-1R or other growth factor receptors. The glioblastoma cells' motility response to all three factors was similar prevented by inhibiting PLC. Importantly, initial studies demonstrated that inhibition of calpain also blocks tumor invasiveness in vitro. Thus, motility per se appears to be the key target rather than any particular molecule.

INVASIVE GROWTH

The definition of invasiveness is the ability of the tumor to grow outside the physiological confines. A number of threads of evidence suggest that this adjacent ectopic growth is likely to be somewhat distinct and probably less stringent than the growth needs for metastatic growth (47). In the main, it has become evident over the past few years that tumorigenesis and progression is a cell field event requiring changes not only in the cancer cell but also in the stromal cells and matrix (48, 49) (Fig. 3). Since invasive growth is contiguous with the orthotopic 'soil', it is conceivable that not only do the carcinoma cells migrate to the ectopic site but also the supporting stromal cells and matrix.

Still, growth outside the matrix barrier is critical, and may involve the same peptide factors that induce ortho-

topic growth (3, 50). These may be the same factors that also promote cell motility. Prostate cancer cell growth relies upon autocrine EGFR signaling, since blocking antibodies limit cell proliferation (45, 51). However, this is distinct from motility signaling, since inhibition of PLC does not limit cell proliferation. Such an autocrine EGFR signaling loop is also required for proliferation of a number of other tumor types such as breast cancer (52). One interesting hypothesis in this regard is that spatially restricted autocrine loops provide for physiologically appropriate cell homeostatic signaling, whereas loss of this restriction generates signals leading to motility (discussed above) and/or proliferation in inappropriate contexts (4). Thus, in this case at least, the same set of signaling elements promotes different cell phenotypes to accomplish two responses needed at distinct stages of invasion.

OPPORTUNITIES AND CHALLENGES

These studies provide a promising start to understanding tumor invasion as the first step towards defining therapies. We have focused on cell motility as a rate-limiting step since it is most clearly distinguishable, whereas the recognition and remodeling steps are intertwined also with motility. It must be emphasized that, at least in experimental models, inhibition of adhesion or extracellular proteases does block invasion (19). However, as growth factor-induced cell motility appears to be a re-creation of fetal and repair biology and distinct from homeostatic and immune response-related motility mediated by adhesion receptors, there seems to be the greatest opportunity for a large therapeutic index.

Much more needs to be understood before one can rationally prevent invasion and turn aggressive tumors into indolent life-long conditions. Advances are likely to be driven by findings that (A) utilize proteomics and post-proteomics to determine the activation status and location of key regulatory switches, (B) define the interplay between adhesion receptors and growth factor receptors in cell motility, and (C) parse the contributions of the non-carcinoma cells in tumor invasion.

These insights that may lead us forward to the next level of understanding will build on the technical advances in imaging and ex vivo organ systems. Tumor cell behavior can now be visualized in live, splayed animals (53–55). Already, such techniques have shown that progressive growth at the ectopic site appears to be the rate-limiting step in metastasis and have also shown active movement of metastatic cells towards blood vessels. Combining these initial forays with multicolored tagging of specific proteins should prove powerful in delineating key molecular functions. However, these model systems are limited by the short time span of observation in h and the difficulty in manipulating the tumors and conditions. As an intermediary step, ex vivo model systems will be key in studying invasion and metastatic seeding. An exciting possibility is a month-long viable liver system that can be visualized continually during this extended period (56). This is particularly germane to ectopic growth studies as the liver is a common site of metastatic growth.

Unfortunately, one key stumbling block that is inhibiting further developments in this field is the lack of a way to study invasion inhibition in people, in clinical trials. Currently, the accepted parameters for Phase II/III trials are geared towards tumor growth and thus are not applicable to therapies that limit progression without a concomitant effect on tumor size. Until clinical oncologists contrive means to determine effectiveness of invasion and metastasis inhibitors, we will not be able to apply this burgeoning knowledge to the benefit of our patients.

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Cutting to the chase: calpain proteases in cell motility

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Calpains are a large family of intracellular proteases whose precise and limited cleavage of specific proteins might be an integral regulatory aspect of signaling pathways. This intriguing mechanism for transducing biochemical and biophysical information from the external milieu seems to operate during cell motility. The two first described and ubiquitous isoforms, μ-calpain and M-calpain, have been implicated in enabling cell spreading by modifying adhesion sites and in promoting locomotion of adherent cells by facilitating rear-end detachment. Recent elucidation of the molecular structure of calpain opens the door for understanding how these pluripotential signal proteins are regulated to help govern migration. Armed with this knowledge, the precise roles of calpains in inflammation, wound repair and tumor progression can be ascertained and offer novel therapeutic targets.

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Our understanding of the calpain family of intracellular cysteine proteases has benefited from intensive characterization in the four decades since their discovery in 1964 [1]. Owing to the limitations of investigative tools, earlier work focused on *in vitro* regulation and activities of these proteases, primarily the two ubiquitous isoforms μ-calpain (calpain I) and M-calpain (calpain II) [2]. Determination of the domain and molecular structures of calpains has provided both mechanistic bases for understanding calpain activation and a platform for deciphering their *in vivo* modulation.

In the past 50 years or so, interest has grown in connecting calpain function to physiology and pathology, and recent studies have focused on its involvement in tissue/organ-level processes such as injury-mediated apoptosis in stroke and ischemia [3], protein degradation in muscular dystrophies [4] and susceptibility for non-insulin-dependent diabetes mellitus [5]. Here, we consider calpain function in an important area of cell biology – that of cell adhesion and migration. This area, of course, has relevance to pathophysiological issues in wound healing, cancer and the immune and inflammatory responses.

Structural considerations

The 13 distinct mammalian calpain gene products identified to date each comprise a large subunit, some of which complex with a single 30-kDa small subunit [6]. Each of the 13 gene products differs in the length of its N-terminal sequence, regulatory domain structures and presence of Ca^{2+}-binding domains; however, all contain the conserved active site. Of the 10 calpains that have been studied at the protein level, the expression patterns vary between tissues, with most of the calpains being relatively cell-type specific. Two ubiquitous calpains, μ-calpain and M-calpain, which have been implicated in adhesion and migration phenomena, have the benefit of being the best characterized owing to their primacy of discovery. These two isoforms were named according to their relative requirement for Ca^{2+} *in vitro*, with μ-calpain requiring micromolar concentrations and M-calpain requiring near millimolar levels of Ca^{2+} to elicit proteolytic activity.

As shown in Fig. 1, the calpain molecule can be divided into five domains, initially conjectured from protein structure/function studies and more recently supported by the crystal structure [7,8]. Domain I contains a short 19-residue N-terminal domain that is cleaved intermolecularly (i.e. by autolysis) either during or following activation. The catalytic domain is divided into two parts, with the active-site cleft formed between them. Domain III is a putative regulatory domain that has been shown to contain sites for phosphorylation (H. Shiraha, pers. commun.) and a phospholipid-binding domain [9]. The fourth domain contains four EF-hand Ca^{2+}-binding domains and is thought to be primarily responsible for the Ca^{2+} requirement shown by calpain.

The elucidation of the crystal structure of M-calpain has shed considerable light on the activation and regulation of calpain. Unlike the papain protease family, which is likely to be the evolutionary precursor of the calpains, the N-terminal domain does not lie in the active site and does not act as a pro-domain. This supports earlier work demonstrating that autolysis of the N-terminus is not required for activation [10,11]. By contrast, the N-terminal domain in the inactive enzyme is contained within a groove in close proximity to the Ca^{2+}-binding domains of both the large and small subunits. The most intriguing aspect of the structure is the active site itself. In the inactive state, the catalytic residues (Cys105, His262, Asn286) are misaligned and too far apart to form a catalytic center [8].

Some conformational change must occur to close and align the active site. Therefore, regulatory events such as Ca^{2+} binding, phospholipid binding, intramolecular cleavage or phosphorylation must effect this alignment. *In vitro*, Ca^{2+}-ion occupancy of each half of the active cleft results in such a change and in subsequent cross-bridging that 'fixes' the cleft in an active state. In addition, release of physical constraints imposed by the three-dimensional

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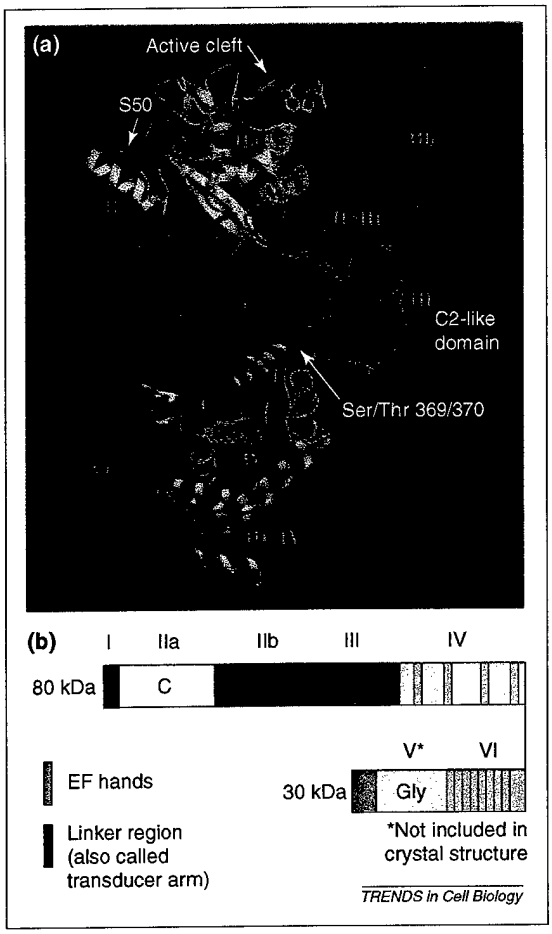

Fig. 1. (a) Ribbon diagram of the crystal structure of human M-calpain in the absence of Ca^{2+}. Adapted, with permission, from Strobl *et al.* [7]. Important regulatory sites discussed in the text are noted with arrows. (b) Schematic representation of the domain structure of the large subunit of M-calpain and μ-calpain (top), with domains color-coded to correspond to the ribbon diagram. The N-terminal domain I is cleaved by intermolecular autolysis before or during activation. The active site is contained within two catalytic domains (IIa and IIb). The putative regulatory domain III contains sites for phosphorylation and phospholipid binding and is followed by a loose linker region, also called the transducer arm. Domain IV contains four EF-hand domains that might contribute to the apparent calcium dependency of calpain and to forming a complex with the small subunit (bottom). The small subunit comprises an N-terminus that is susceptible to proteolysis, a glycine-rich domain and a Ca^{2+}-binding domain highly homologous to domain IV of the large subunit.

Table 1. Select calpain targets – proteins involved in motility and adhesion

Target	Cellular locale	*In vivo*[a]	*In vitro*	Refs
EGFR	Plasma membrane	Yes	Yes	[50]
Talin	Adhesion complex	Yes	Yes	[20,21,51–53]
Ezrin	Adhesion complex	Yes	Yes	[31,54]
Paxillin	Adhesion complex	Yes	–	[55]
Vinculin	Adhesion complex	Yes	–	[56]
Spectrin	Adhesion complex	–	Yes	[57,58]
Filamin	Adhesion complex	–	Yes	[59]
α-Actinin	Adhesion complex	Yes	No	[60,61]
Integrin-β1	Adhesion complex	Yes	Yes	[62]
Integrin-β3	Adhesion complex	Yes	Yes	[17,62]
Integrin-β4	Adhesion complex	Yes	–	[63]
Tau	Pan-cellular	Yes	Yes	[64,65]
MAP2	Pan-cellular	–	Yes	[66,67]
FAK	Adhesion complex	Yes	Yes	[55,68,69]
pp60Src	Adhesion complex	Yes	–	[26]
PKC	Pan-cellular	Yes	Yes	[56,70]
RhoA	Pan-cellular	Yes	–	–[b]
MLCK	Pan-cellular	–	Yes	[71,72]

[a]Cell culture.
[b]J.E. Fox, pers. commun.
Abbreviations: EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; MAP2, microtubule-associated protein 2; MLCK, myosin light chain kinase; PKC, protein kinase C.

structure would also be required (Z. Jia, pers. commun.). How this activating intramolecular reorganization is effected *in vivo* is a major challenge in determining the physiological roles of calpains.

Activation and regulation

Multiple, potentially alternative or complementary mechanisms of activation and regulation have been suggested for the ubiquitous calpain molecules. Extrapolating from *in vitro* findings, it was presumed at first that calpains are activated by intracellular Ca^{2+} fluxes. Although some indication that Ca^{2+} levels high enough for μ-calpain activation could be achieved in highly localized Ca^{2+} puffs (up to ~600 nM in non-excitable cells) or sparks (excitable cells) [12], these Ca^{2+}-release events have not generally been observed during normal cell homeostasis and therefore might have limited involvement in cell signaling. In addition, the *in vitro* Ca^{2+} levels required for M-calpain activation appear to be generally unattainable under physiological conditions, with the exception of events related to cell death [13,14]. Therefore, several mechanisms have been suggested either to lower the Ca^{2+} requirement or to substitute for Ca^{2+} altogether. These include phospholipid binding, autolysis, release of calpain from its inhibitor calpastatin, binding of activator proteins and phosphorylation (Box 1). These studies have mainly focused on calpain behavior *in vitro*, which could well be significantly different from its behavior and function *in vivo*.

Whatever mechanism(s) is/are used to effectively reconstitute the active site and create an active molecule, one must not lose sight of the fact that the upstream signals controlling this activation, and the downstream targets of calpain activity, are just as important in understanding the physiological role(s) of this enigmatic protease. Furthermore, the modes for activating calpains might vary not only between isoforms, such as Ca^{2+} fluxes operative for μ- but not M-calpain (see below), but might also depend on subcellular localization and/or cell response – as the ubiquitous isoforms are found throughout the cell, including in the nucleus, and have been implicated in responses as diverse as proliferation and migration.

Calpain as a signal transducer for cell migration

Calpain activity has been shown to be crucial for a diverse spectrum of cellular responses, at least some of which are mutually exclusive [15]. These include apoptosis, proliferation, protein turnover and, most recently, cell adhesion and motility. Although the specificity of some of these responses is considered to be dictated by the specific calpain isoforms, most can be accomplished by the two ubiquitous isoforms, μ- and M-calpain. Further confounding the simple assignment of cell response to molecular effector is the fact that both of these isoforms are present in multiple subcellular locations. Thus, in dissecting the role of calpain-mediated proteolysis in cell adhesion and motility, one has to be cognizant of the spatial and probably the temporal nature of calpain activation.

Spatial constraints on calpain activation are even more crucial when one considers through what mechanism calpain modulates cell adhesion and motility. Although this mechanism is still unclear, all evidence supports a proteolytic event. Calpain has many target molecules *in vivo*, including many proteins found in adhesion complexes – but also other cytosolic proteins. M-calpain has been observed to colocalize with talin in focal adhesions in some cell types [16]; however, this has not been seen in all cell types [17]. This suggests that the targets important for adhesion and motility are probably only a subset of the total number of calpain targets (see Table 1). Even though one can propose plausible and testable models for localizing calpain activation to the internal face of adhesion complexes, this still leaves a large number of attractive targets, including talin, paxillin, α-actinin, ezrin, focal adhesion kinase (FAK) and the cytosolic tails of β1 and β3 integrins. All these molecules are present in focal adhesions [18]. Accordingly, it is conceivable that any given target might be sufficient, but not necessary, for the weakening of integrin linkages to the substratum that is rate limiting for tail detachment [19].

Elucidation of the key calpain target(s), and of the subsequent mechanism for the regulated disassembly of adhesion sites, is likely to uncover novel modes of signaling and molecular regulation. Further complicating the picture is the observation that calpain proteolyses at limited sites in each molecule,

Box 1. Proposed activation mechanisms for calpains

Phospholipid binding

Binding of phospholipids decreases the Ca^{2+} requirement for calpain activation *in vitro* [a,b]. Furthermore, calpain translocates to the plasma membrane in the presence of Ca^{2+}, where it associates with phosphatidylinositol (4,5)-bisphosphate [c]. As there is a putative phospholipid-binding domain in the regulatory domain of calpain [d], this needs further investigation. This domain is particularly relevant to the effects on motility and adhesion as calpain functions at the inner face of the plasma membrane during these cell responses (see below).

Autolysis

Immediately upon calpain activation, autolyzed fragments of 76 and 78 kDa appear, and it is because of this that autolysis has been the most investigated mechanism suggested to replace the need for high Ca^{2+} levels. To yield these fragments, calpain cleaves, respectively, 18 and 26 amino acids from the N-terminus of its large subunit. The shortened subunit possesses catalytic activity [e] and requires a lower Ca^{2+} concentration for activation than the intact one [f,g]. Nevertheless, the Ca^{2+} requirement for autolysis, even in the presence of phospholipids, is much greater than physiological levels, begging the question of whether Ca^{2+} is required for autolysis *in vivo*. Earlier studies showed that the intact large subunit [h–k] and a mutant incapable of autolysis [l] both possess catalytic activity. Thus, the removal of these 18/26 amino acids might make the activation irreversible or generate a signal for attenuative degradation.

Escape from endogenous inhibition

All cells express an endogenous inhibitor of calpains – calpastatin – which binds to and inactivates calpains through each of its four repetitive inhibitory domains. However, although release of calpain from calpastatin correlates with activity, it is not sufficient for activation. Furthermore, calpastatin is neither always present in excess molar levels nor always colocalizes with calpain. Moreover, Ca^{2+} fluxes enhance calpastatin inhibition of calpains, suggesting that calpastatin might function to attenuate activated calpains rather than tonically prevent calpain activity [m,n]. Despite the conflicting evidence of physiological relevance, overexpression of this molecule can be employed to prevent calpain activation.

Protein coactivators

It has been proposed that certain protein–protein interactions alter calpain activity. Although it has been suggested that dissociation of the large subunits from the small subunit is an activation mechanism, this remains controversial. Select proteins copurify with active calpains, which increases the autolysis of calpains *in vitro*. Possible candidates for these copurified proteins have been found in some cell types. In rat skeletal muscle, bovine and rat brain, activator proteins for μ-calpain were found that increased autolysis and lowered the Ca^{2+} requirement [o–r]. An activator for M-calpain, found in rat skeletal muscle, has been identified as acyl-CoA-binding protein [s]. Unfortunately, the association and activation of calpain *in vivo* by these proteins has not been demonstrated, and the mechanism by which they could activate calpains is unclear.

Phosphorylation

Most recently, old standbys of signal transduction – phosphorylation cascades – have been proposed as being involved in activating and attenuating M-calpain. Early reports were contradictory in that calpains were shown to be phosphorylated *in vitro* but not *in vivo*, as determined by autoradiography [t,u]. However, with technological advances, both M- and μ-calpain have been shown

resulting in long-lived specific fragments. That these fragments might be activated or serve as dominant-negatives rather than simply being removed by degradation is strongly suggested by the observation that the talin calpain cleavage product has a higher affinity for binding to β3 integrin cytoplasmic tails than intact talin and subsequently regulates integrin activation [20,21]. Calpain can therefore act as a signaling molecule, but it elicits different effects depending on which target molecules are cleaved and perhaps on the relative levels of their cleavage. Indeed, when approaching the subject of the involvement of calpain in cell adhesion and motility, it is necessary to keep in mind that the activity of calpain noted (or, experimentally, the inhibition of calpain activity) will probably indicate cleavage of physiologically relevant calpain targets; however, perhaps the target will vary or will involve different relative amounts.

Cell adhesion

Initial studies of possible roles for calpain focused on cell adhesion in platelets, an interesting system in which calpain clearly plays a role in secretion, adhesion and aggregation. Inhibition of calpain using an overexpressed form of calpastatin prevents thrombin-stimulated α-granule secretion, platelet aggregation and cell spreading on glass surfaces [22]. Platelets have a unique advantage over other cell types for studying calpain function in that they predominantly express μ-calpain, having negligible levels of M-calpain [15]. Therefore, molecular inhibition of μ-calpain (for example, with dominant-negative μ-calpain) is sufficient to downregulate all detectable calpain activity, and antibodies to the autolyzed form can yield meaningful results. It is necessary to note, however, that, although calpain activity is no longer detectable using traditional techniques, platelet function in a mouse μ-calpain knockout model presents surprisingly minor deficits [23].

In this context, calpain was shown to be part of the integrin signal-transduction apparatus. Calpain is activated following signaling across the platelet integrins αIIbβ3. Integrin binding to fibrinogen in the presence of thrombin activates calpain, with the subsequent appearance of autolyzed μ-calpain and calpain cleavage products of talin, a protein that

to be phosphorylated *in vivo* (J.Y. Cong, V.F. Thompson and D.E. Goll, unpublished). Under unstimulated conditions, there are three sites each of phosphotyrosine, phosphoserine and phosphothreonine phosphorylation, with calpains isolated from a variety of tissues demonstrating varied substoichiometric phosphorylation. We also have reported that growth factors activate M-calpain downstream of ERK/MAP kinase [v] and have now found evidence suggesting that this occurs at Ser50. This is intriguing as muscle-specific calpain III – which does not require increased Ca^{2+} [w] – presents a glutamic acid residue at this site. Further support for phosphorylation as a modulatory mechanism is offered by our finding that cAMP-dependent protein kinase A phosphorylation of Ser369/Thr370 in M-calpain inhibits calpain activity by rigidifying the structure in an open, inactivating conformation (H. Shiraha, A. Golding, Z. Jia, J. Chou and A. Wells, unpublished). All these data suggest that a complex pattern of phosphorylation might regulate M-calpain activation and inactivation.

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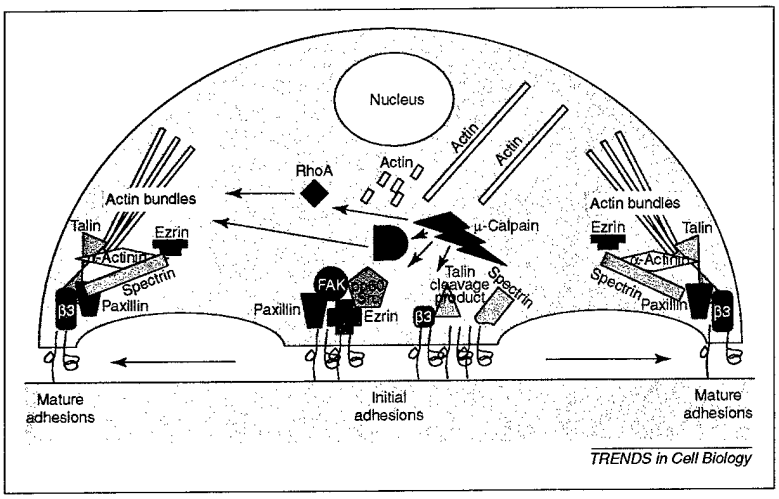

Fig. 2. Possible molecular interactions in a spreading cell. Spreading cells initially exhibit formation of newly defined integrin-containing clusters, which contain calpain and cleaved talin, spectrin and β3 integrin (shown in cell center for clarity, these early adhesions are actually observed at the cell periphery). Early activation of the Rac GTPase leads to formation of focal clusters and later to Rho-mediated formation of new focal adhesions and stress fibers (actin bundles). These later-formed structures do not contain calpain or calpain cleavage products as seen in bovine aortic endothelial cells [17].

links integrins to the cytoskeleton. This activation is inhibited by addition of the integrin competitive inhibitor peptide RGDS (single-letter amino acid code) [24,25] and by monoclonal antibodies to the αIIbβ3 integrin [24]. These studies also demonstrated an integrin-dependent translocation of calpain to the peri-plasma membrane space [24], suggesting that integrin-mediated signals target calpain to focal adhesions, where many calpain substrates are located. Calpain associates with focal adhesion proteins in platelets [24], regulates the attachment of αIIbβ3 to the cytoskeleton and relaxes the retraction of fibrin clots [26]. The mechanisms of these actions are unclear. Activation of calpain by both thrombin and the Ca^{2+} ionophore A23187 increased the proteolysis by calpain of $pp60^{c\text{-}src}$ and phosphotyrosine phosphatase-1B, which then dissociated from the cytoskeleton and became inactive. This correlated with the inhibition of fibrin-clot retraction observed in aggregated platelets in the presence of Ca^{2+}.

Calpain inhibition blocked cleavage of the actin-binding protein talin, indicating that talin is a calpain substrate, whereas calpain activation caused the movement of both cleaved talin and integrin αIIbβ3 from the Triton-X-100-insoluble fraction (cytoskeleton) to the Triton-X-100-soluble fraction (cytosol) [26]. Calpain therefore functions as a signaling molecule in platelets, regulating the cellular response of thrombin-stimulated aggregation and clot formation.

Cell spreading

Cell spreading is a complicated phenomenon that requires active remodeling of adhesion sites to enable cells to extend processes subsequent to attachment (Fig. 2). In its simplest form, it might be considered similar to platelet aggregation driven by integrin activation. It is also considered somewhat similar to the process of forward protrusion during active cell locomotion. In bovine aortic endothelial cells, calpain is required for cell spreading and affects processes such as adhesion induced by the GTPase Rac, promoting extension and the requisite remodeling of cell adhesions to enable further extension [17,27]. Inhibition of calpain by calpeptin or the compound MDL28170 in cells plated on fibronectin caused a marked reduction in cell spreading and focal adhesion formation, without affecting initial attachment. Cells that were allowed to spread and then exposed to cell-permeant calpain inhibitors became rounded and lost focal adhesions and stress fibers. The observation that spreading was specifically dependent on the activity of μ-calpain was reinforced by the observation that overexpression of μ-calpain led to overspread cells with excessive focal complexes, focal adhesions and stress fibers. Cells that were transfected with a dominant-negative μ-calpain, in which the active site histidine residue was mutated to alanine, became rounded and lost stress fibers and actin networks [27].

Calpain acting to enable assembly of cell adhesions required for attachment is also found in T cells, where integrin ligation activates calpain to promote integrin diffusion, formation of focal complexes and, ultimately, cell spreading [28,29]. Although the molecular signals that activate calpain in these processes are unknown, specific interventions found that this integrin-based spreading was mediated by μ-calpain.

Because focal adhesion and stress-fiber formation are processes known to be dependent on the Rho family of GTPases, calpain has been postulated to regulate these proteins. Calpain could well be an upstream signaler of Rac and Rho activation as expression of active Rac or Rho constructs overcomes the effects of calpain inhibition on adhesion and stress fiber formation [27]. In addition, recent work has proposed a model by which calpain activates Rac (presumably indirectly) very early in spreading cells, producing small integrin-containing adhesions that contain calpain-cleavage products of talin, spectrin and β3 integrin. Calpain might activate Rac in these adhesions, triggering a cascade of spreading events [17]. This fits well with studies showing that calpain is required for lamellipodial extension and filopodia formation as these processes also have been associated with the Rho GTPases. Calpastatin overexpression in NIH3T3 fibroblast cells inhibits calpain activity, functionally blocking cell spreading; and these cells lack lamellipodia and motility. However, unlike the situation in bovine aortic endothelial cells, these cells have large numbers of stress fibers, abnormal filopodia and abnormal retraction fibers [30]. Interestingly, these cells had higher levels of intact ezrin, a β-actin-binding protein involved in cytoskeletal reorganization [31]. Calpains

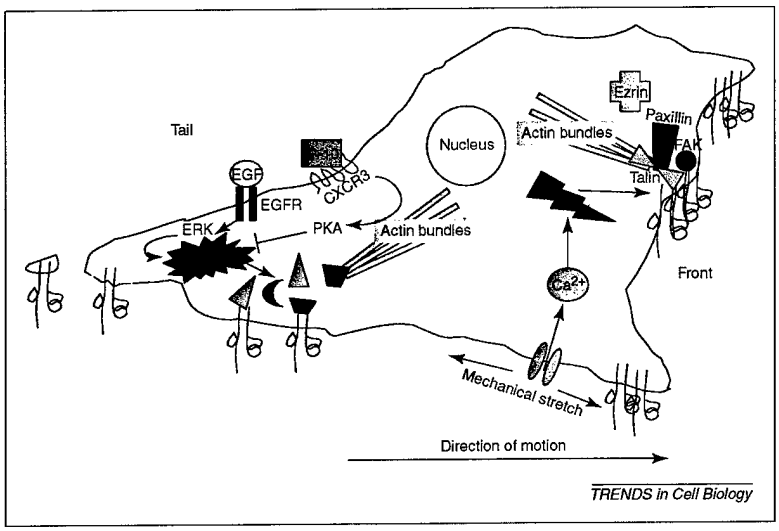

Fig. 3. Model of differential calpain isoform activation in a migrating cell. μ-Calpain acts at the cell front, activated by integrin signaling and/or mechanically transduced signals that activate stretch-activated Ca^{2+} channels. μ-Calpain then cleaves its target proteins, including talin, ezrin, focal adhesion kinase (FAK) and the cytoplasmic tail of β1 and β3 integrins, to facilitate adhesion turnover and formation (see Table 1). During haptokinesis, μ-calpain might also act at the rear, triggered by mechanical signals [34,35]. At the cell rear, M-calpain is activated by growth-factor-receptor signaling (i.e. by the EGFR) at the cell membrane, and M-calpain is potentially retained at the membrane by phospholipid binding [40,42]. M-calpain acts on its targets to disrupt the intracellular face of the adhesion, allowing release of the integrins and the appearance of a trail of integrin patches behind the cell. Counter-regulatory signals, such as the protein kinase A-mediated signal from the chemokine IP-10, act to downregulate M-calpain and prevent cell migration [41].

cleave the cytosolic tail of ezrin, and this suggests that, in spreading cells, calpain might prevent ezrin from binding to actin and thus allow cytoskeletal reorganization [30].

Adhesion-related rear detachment during motility

That calpain-mediated regulation of cell–substratum adhesion should be crucial not only during spreading and forward protrusion but also during rear release (which enables a cell to productively move from its initial site) is not immediately obvious [32,33]. However, in light of the apparent ability of calpain to functionally affect many adhesion proteins by limited cleavage, it is not unreasonable to propose that calpain is not strictly involved in adhesion formation but functions to increase adhesion turnover, with cellular conditions dictating whether this would have an adhesive or releasing consequence. Indeed, concurrent with the above studies on cell spreading, haptokinetic motility – motility that occurs when cells are plated on an activating substrate and mediated primarily by integrins – was shown to be calpain dependent. Migration on fibronectin of CHO cells transfected with both β1 and β3 integrins was sensitive to calpain inhibition by calpain inhibitor (CI)-1, CI-2 and BDK (benzyloxycarbonyl-Leu-Leu-Tyr diazomethyl ketone) [34]. Calpain inhibition stabilized peripheral focal adhesions, increased adhesiveness and decreased the detachment rate; again contrary to expectations derived from cell-spreading studies. However, if the effect of calpain was to alter adhesion to substratum, one would predict a varied effect dependent on substrate density. The reduction in motility was dependent on substrate concentrations, with cells migrating on lower levels of substrate less sensitive to calpain inhibition. High substrate concentrations, where the cell is more tightly adherent, increased the sensitivity to calpain inhibition [35]; non-motile cells were quite extended, with long tail processes. Together with the decrease in detachment rate, this suggests that calpain is required for rear detachment during haptokinetic motility. Calpain modulated cell motility in a manner dependent on adhesive strength, which was identical to alterations in integrin affinity for fibronectin [36]. This might indicate that calpain is acting as a physiologic 'rheostat' for adhesion control. It is interesting to note that the activation of calpain mediated by fibrinogen binding to the αIIbβ3 integrin transfected into the CHO cells might have paralleled the more normal physiological activation by the same integrin in platelets noted earlier.

During motility, cells must both form new adhesions at the leading edge (similar to cell spreading) and also dissolve adhesions at the cell rear to allow forward movement. When one considers the studies described above as a whole, an apparent conflict exists: how does calpain regulate both adhesion formation and disruption within the same cell? Calpains, having been implicated in both adhesion formation (cell spreading) and adhesion disruption (rear release), must therefore be regulated differentially in a front-versus-rear fashion. This asymmetry of action might be mediated through asymmetric localization of calpain isoforms, spatial and/or temporal asymmetry of activation mechanisms (see below) or asymmetrical localization or distribution of targets. At the very least, it seems likely that calpain functions to regulate adhesion turnover, rather than specifically to generate or breakdown adhesions.

Exactly how calpain is regulated during haptokinesis remains unknown, but a rough model is emerging. In the above experiments, Huttenlocher and colleagues [34] found that cell variants that expressed low levels of μ-calpain behaved identically to those exposed to inhibitors, despite having a larger proportion of M-calpain, suggesting an isoform-specific activating mechanism. Interestingly, movement of cells on higher fibronectin densities requires myosin light chain kinase-dependent contraction [37], and increases in intracellular Ca^{2+} levels are seen in highly stretched and contracting cells during fibroblastoid movement [38]. Neither of these studies implicated calpain activity, but it is plausible that such mechanical stretching of the membrane might lead to activation of stretch-activated Ca^{2+} channels, which would contribute to the activating mechanism of μ-calpain action.

Growth-factor-induced cell de-adhesion

The apparent sufficiency of the μ-calpain isoform for cell spreading and integrin-mediated adhesion and migration raises a question regarding the role of the M isoform, especially as all studies suggest that they have the same apparent substrate specificity. One simple explanation, of course, is that this would represent merely a safeguarding redundancy. However, a growing body of evidence indicates that the answer is likely to be more complex. Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) stimulate motility by mechanisms imposed upon the underlying haptokinesis [33]. We found chemokinesis (i.e. growth-factor-mediated motility) also requires de-adhesion [39], dependent on calpain, as inhibition with either calpeptin or CI-1 blocks EGF-induced de-adhesion and cell migration [40]. Interestingly, this de-adhesion and motility occurs through extracellular regulated kinase (ERK/MAP kinase) phosphorylation and activation of M- but not μ-calpain, in the absence of a Ca^{2+} flux [40] (A. Glading, I.J. Reynolds, H. Shiraha and A. Wells, unpublished). The site of phosphorylation appears to be Ser50, which is absent in μ-calpain. This provides a rationale for the evolutionary duplication of the ubiquitous isoforms, to enable identical biophysical processes to be accomplished by disparate signals that converge only at the final point of adhesion disassembly (Fig. 3).

Both EGF receptor (EGFR) signaling and EGFR activation of ERK occur throughout the cell, with ERK phosphorylating targets in both the cytosol and the nucleus. As this pathway activates M-calpain, the question then arises as to how M-calpain activity is targeted to the relevant intracellular locations. Inhibition of M-calpain causes cells to adopt an extended morphology, with long, elongated tails [41]. This suggests that, as was shown for integrin-mediated motility above, growth-factor-mediated M-calpain activation is required for tail detachment. Therefore, M-calpain activation needs to be asymmetric to enable progressive locomotion and not trigger front detachment or cell responses other than motility. We have found that EGF induces calpain activity only when both the EGFR and ERK are associated with the plasma membrane [42]. This would put active M-calpain in proximity to the cell membrane and putative targets in the adhesion complex. Intriguingly, we have found that phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] is depleted in the lamellipodia during active motility (J. Chou and A. Wells, unpublished), confirming the rapid inositol trisphosphate turnover seen to reinforce chemotactic gradients [43]. This presents a model in which M-calpain is targeted to the cell membrane through localization of EGFR and ERK signaling, where it might bind to PtdIns(4,5)P_2. The observed asymmetry of phospholipids would provide for spatially targeted calpain proteolysis away from the front and towards the rear – the locale targeted for de-adhesion.

Physiological and pathological roles

Calpain performs vital operations in cell motility both by labilizing cell–substratum adhesions to allow forward extension and rear release, and by possibly acting as a positive signal to modulate functioning of the Rho GTPases. The utility of targeting calpain and its modulatory pathways depends on how these are integrated in the development and homeostasis of organisms. As the prominence of calpain-mediated adhesion modulation varies with substratum composition [35], it is not *a priori* evident that these would be productive manipulations. If, however, the functions of calpain are or can be restricted *in vivo*, it could become an exciting new target for pharmacological agents.

M-calpain as a target during wound repair

Growth-factor-induced cell motility is considered crucial for organogenesis, and especially so for regenerative wound repair. We have identified calpain as a potentially crucial target to limit fibroplasia late in the regenerative phase of skin repair [41]. Two angiostatic chemokines of the CXC family – interferon-γ-inducible protein-10 (IP-10) and IP-9 – are produced from the neovasculature and redifferentiated keratinocytes, respectively. Acting through their common receptor, CXCR3, they trigger protein kinase A phosphorylation of Ser369/Thr370 in M-calpain to prevent growth-factor-induced calpain activity and motility in dermal fibroblasts (H. Shiraha, A. Glading, Z. Jia, J. Chou and A. Wells, unpublished).

In mesenchymally transitioned keratinocytes that require active migration to re-epithelialize the wound, however, IP-9 and IP-10 appear to promote motility (L. Satish, A. Glading, H. Shiraha, D. Yager and A. Wells, unpublished). Preliminary observations suggest that this is probably accomplished through an IP3-initiated Ca^{2+} flux, activating μ-calpain in these cells. How opposite outcomes are actuated by the same receptor upon the two isoforms is unknown; however, it probably involves differential utilization of heterotrimeric G protein isoforms that couple to adenylate cyclase (inhibition of M-calpain) or phospholipase-Cβ (activation of μ-calpain). Thus, the different calpain isoforms seem to function as divergently regulated rate-limiting molecules to effect cell motility in opposing fashions dependent on cell type.

Interestingly, blocking de-adhesion can be considered a 'gain of function' rather than an inhibitory action in some circumstances. During the late resolving phase of wound repair, fibroblasts switch from reconstitution to reorganization, a process that includes contraction of the dermal matrix. Inhibiting calpain-mediated de-adhesion is proposed to channel the growth-factor-induced fibroblasts to cause contraction of a collagen lattice. It could do this by efficiently transferring to the matrix the intracellular contractile force necessary to detach the tail and move the cell body forward during

motility [73]. In such a setting, calpain operates as a molecular switch between two alternative cell functions, highlighting the central signaling role of these molecules.

Other integrative roles

Calpain activity appears central to adhesion and movement of cells of the immune response [28,29]. Thus, many have speculated that calpain could be targeted to modulate the immune response. However, this has yet to be directly addressed either in *in vivo* or *in vitro* networks, as with wound repair, above. One might focus on the initial stages of adhesive recognition of inflammatory sites and subsequent spreading to give access to the interstitial space [44]. The possible involvement of calpain-mediated de-adhesion in further motility is open to question as such cells appear to use reversible detachment mechanisms, operating at much lower adhesion regimens, although the recycling of integrins requires Ca^{2+} transients [45].

Calpain-mediated motility processes might also be productively targeted to limit tumor spread. Active cell movement is required by tumor cells during both invasion and metastasis and by endothelial cells during reactive angiogenesis [46,47]. Further investigations to delineate whether calpain-mediated attachment and motility are rate limiting in tumor progression and immune responses might add additional targets to our therapeutic arsenal.

Future considerations

Progress in understanding calpain function should accelerate swiftly in the next few years as investigators integrate the detailed biochemical and structural information with emerging cell-biological, organismal and proteomic capabilities. For instance, the current evidence for spatial control of calpain function during EGF-induced motility is only a first-level crude analysis. New imaging methods could shed light on molecular spacing and subdomains within seemingly homogenous locales (e.g. front–rear asymmetry and molecular positioning within adhesion complexes). In addition, the temporal nature of calpain activation and functioning could be appreciated by live-cell imaging, using proteolytically activated reporters. This might be controlled, in part, during productive migration, at the transcriptional level (of which precious little is known with respect to motility – except for the observation that there appears to be a compensatory upregulation of protein production in response to growth-factor-induced turnover [40]). Proteomics holds the promise of defining physiologically relevant calpain targets for calpain activity in cell adhesion and motility.

Placing calpain function in physiological and pathological contexts requires analyses in tissue systems and animal models. *Ex vivo* organ systems are still in their infancy but should see quantifiable advances in the next few years [48]. These situations would enable coupling of directed manipulations to real-time imaging capabilities.

Model animal systems are being established, but certain limitations need to be overcome. Genetic ablation of the common small subunit, eliminating the ubiquitous calpains, results in lethality in early- to mid-gestation [49]. Cell proliferation in rescued fibroblasts is relatively unaffected, although cell adhesion and motility appear impaired (A. Huttenlocher, pers. commun.). Still, temporal and tissue-specific ablation of calpain and of individual isoforms is required to tease out individual physiological roles.

These exciting findings will need to be integrated with other aspects of cell motility and adhesion, and with other regulators of such processes – and also with the various other roles of calpains. Only then will we be able to derive testable models that should uncover new biological principles, such as the emerging idea of limited proteolysis as a signal-transduction process, and also define new targets for interventions and bioengineering.

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